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Sihler's whole mount nerve staining technique: a review

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Abstract

Sihler's stain is a whole mount nerve staining technique that renders other soft tissue translucent or transparent while staining the nerves. It permits mapping of entire nerve supply patterns of organs, skeletal muscles, mucosa, skin, and other structures after the specimens are fixed in neutralized formalin, macerated in potassium hydroxide, decalcified in acetic acid, stained in Ehrlich's hematoxylin, destained in acetic acid, and cleared in glycerin. The unique advantage of Sihler's stain over other anatomical methods is that all the nerves within the stained specimen can be visualized in their three-dimensional positions. To date, Sihler's stain is the best tool for demonstrating the precise intramuscular branching and distribution patterns of skeletal muscles, which are important not only for anatomists, but also for physiologists and clinicians. Advanced knowledge of the neural structures within mammalian skeletal muscles is critical for understanding muscle functions, performing electrophysiological experiments and developing novel neurosurgical techniques. In this review, Sihler's stain is described in detail and its use in nerve mapping is surveyed. Special emphasis is placed on staining procedures and troubleshooting, strengths and limitations, applications, major contributions to neuroscience, physiological and clinical significance, and areas for further technical improvement that deserve future research.

Keywords

innervation; intramuscular nerve branching; mucosa; nerve distribution; nerve mapping; nerve staining; neuroanatomy; peripheral nerves; Sihler's stain; skeletal muscles; troubleshooting; whole mount

Sihler's stain is a whole mount nerve staining technique. It is the only method available for mapping the entire nerve supply patterns of mammalian skeletal muscles, mucosa, and skin. This technique has a long history, a specific staining mechanism, and distinct advantages over other methods.

Brief history

Sihler's stain is a largely forgotten technique with a long history. It was introduced late in the nineteenth century by Sihler (1895) for identifying neuromuscular spindles. Almost half a century later, Sihler's stain was modified by Wharton (1937) and Williams (1943) for

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investigating the innervation of the kidney, uterus, and ovary in humans (Wharton 1937) and the leg and food of the amphibian, *Triturus viridescens* (Williams 1943). Some investigators (Freihofer 1966, Puzdrowski 1987) used this technique to stain nerves in fish. Sihler's stain was modified further late in the twentieth century by Liem and Van Willigen (1988) and Wu and Sanders (1992) to demonstrate the distribution of peripheral nerves in whole mount preparations. Since then, the modified Sihler's nerve staining technique has been used increasingly to investigate the innervation of skeletal muscles and mucosa in mammals.

Staining protocols

The following six solutions and eight process steps are required for modified Sihler's nerve staining technique.

Staining solutions

1. 10% un-neutralized formalin.
2. 3% aqueous potassium hydroxide (KOH) solution (add three drops of 3% v/v hydrogen peroxide to 100 ml 3% w/v KOH solution for depigmentation).
3. Sihler's solution I (one volume glacial acetic acid, one volume glycerin, and six volumes 1% w/v aqueous chloral hydrate).
4. Sihler's solution II (one volume stock Ehrlich's hematoxylin, one volume glycerin, and six volumes 1% w/v aqueous chloral hydrate).
5. Lithium carbonate solution 0.05% w/v.
6. Aqueous glycerin 50% v/v and 100% glycerin.

Staining steps

Fixation—The harvested specimen is washed with running tap water, then fixed in 10% un-neutralized formalin. The duration of this step depends on the specimen size. Adult human larynx, pharynx, and tongue must be fixed for at least four weeks. The formalin fixing solution may be changed once a week or when it becomes cloudy.

Maceration and depigmentation—The fixed specimen is washed under running tap water for about 1 h, then immersed in 3% KOH solution for maceration and depigmentation. The duration of this step varies from several days to several weeks depending on the sample size. For adult human larynx and pharynx, it requires three weeks. The solution should be changed twice a week or whenever it becomes cloudy or dark brown. During this period, the specimen is gradually whitened. The end point of this step is when the specimen becomes translucent and the nerves, especially the small nerve branches, can be seen clearly as white fibers.

Decalcification—The well-macerated and depigmented specimen is washed under gentle running tap water for about 1 h, then placed in Sihler's solution I to decalcify the specimen. The solution should be changed twice a week. The duration of this step varies with different whole mounts. For adult human larynx and pharynx, it requires 2–3 weeks.

Staining—The decalcified specimen is washed under gentle running tap water for about 30 min, then stained in Sihler's solution II. Total time for staining varies with the thickness of the specimen. Adult human larynx and pharynx usually are stained for four weeks. During staining, the solution may be changed once or twice if the solution changes from dark blue to light purple. The end point is when all nerves within the specimen are stained dark violet blue and the finest twigs can be seen under a dissecting microscope.

Destaining—The stained specimen is washed under gentle running tap water for about 30 min, then destained in Sihler's solution I with agitation. Total time for destaining also depends on the specimen size. For adult human larynx and pharynx, it requires about 3–4 h. The solution should be changed whenever it turns blue or purple. During destaining, the specimen should be checked once an hour by transillumination under a dissecting microscope. This step is stopped when the nerve branches are dark blue or violet and the finest twigs can be seen clearly, whereas the muscle fibers and other non-nervous tissues have a transparent lavender color.

Neutralization—After destaining, the tissue is acidic and must be neutralized with freshly made 0.05% lithium carbonate solution. The specimen is washed under gentle running tap water for about 1 h, then neutralized with agitation for 1–2 h to darken the color of the nerves. The specimen should be checked every 30 min and the solution should be changed if it turns pinkish. The end point of this step is when the color of the nerves changes from purple to deep blue.

Clearing—The well-neutralized specimen is washed under gentle running tap water for 1 h, then cleared either in dilute aqueous glycerin at sequential concentrations (i.e., 40, 60, and 80%; 24 h each) or in 50% aqueous glycerin for 3–5 days to clear excess stain. During this period, the specimen should be checked every day. This step should be ended when the finest nerve twigs can be seen clearly under a dissecting microscope after the specimen is transilluminated.

Transparency—The cleared specimen is preserved in 100% glycerin with a few thymol crystals for transparency. The pure glycerin with thymol crystals may be changed every six months or perhaps longer.

Rationale, mechanisms, end results

A whole organ or specimen processed with Sihler's stain is softened in potassium hydroxide, decalcified in acetic acid, stained in Ehrlich's hematoxylin, destained in acetic acid, and cleared in glycerin. This technique stains axons and renders non-nervous tissues transparent, thereby making the nerve branches visible in whole mount preparations. Only the myelin in neural tissue is stained by the hematoxylin of Sihler's stain (Liem and Van Willigen 1988).

Modified Sihler's stain renders a whole organ or specimen relatively translucent, while counterstaining its nerve supply. In a well-stained specimen, all nerves are stained dark blue, whereas non-nervous tissues are transparent. The Sihler's stained complete nerve map allows all nerve branches to be visible to their terminals so that the morphological relation between the muscle fibers and the nerves can be seen three dimensionally.

Troubleshooting technical issues

Tissue sampling

Accurate data concerning the innervation of a target organ or tissue are dependent on not only good staining, but also complete harvesting of the specimen with its innervating nerve(s). Before removing an organ or muscle, some important anatomical landmarks may be labeled and morphometric measurements made. While a sample is being removed, the main trunk(s) of its innervating nerve(s) should be identified microscopically and tagged with sutures to ensure complete tissue sampling. After extramuscular identification and labeling of the nerve (s) supplying the muscle to be examined, the muscle should be excised *en bloc*. This is emphasized because the entire nerve supply cannot be determined in an incomplete tissue sample. If two or more nerves supply an organ or muscle, microdissection of the stained specimen cannot differentiate which branches come from a given nerve in an incomplete

sample. The pharynx is a good example, because it receives its innervation from several nerves from the pharyngeal plexus including the pharyngeal branches of the vagus and glossopharyngeal nerves, external superior laryngeal nerve, and sympathetic nerve fibers from the superior cervical sympathetic ganglion. To determine the distribution of these nerves in the pharynx, cranial nerves X and IX must be transected at the jugular foramen where they emerge from the skull (Fig. 1). Otherwise, these nerves cannot be differentiated correctly even though they are stained well (Fig. 2).

Staining-related items

A well stained specimen is crucial for mapping its nerve supply. To this end, a number of factors influencing nerve staining must be taken into account.

Container—Each specimen should be placed in a relatively large container to ensure enough space around the specimen. For an adult human tongue or larynx/pharynx, a 10 × 15 cm plastic container usually is used.

Tissue fixation—Adequate tissue fixation, which is essential for good staining, takes one month or longer for a larger whole mount specimen. Although immersion fixation of fresh specimens commonly is used and works for human and animal tissues, adequate tissue fixation may be achieved by perfusion if animal tissues are studied.

Hematoxylin solution—The hematoxylin solution is important for nerve staining. An effective working solution must be at least three weeks old and not older than six months. The time is emphasized, because it tends to understain if not ripe enough and overstain if too old. It is difficult to remove excess stain from an over-stained specimen during the destaining step. In addition, the amount of hematoxylin solution may be decreased if the specimen is small and easily over-stained.

Timing—There is no fixed timetable for each of the staining steps. The period for each step depends largely on the size and type of the tissue sample; a longer time is required for larger specimens. In addition, the use of agitation shortens the duration of the process. It should be pointed out, however, that the hematoxylin staining step with agitation is not recommended, because the smaller and thinner specimens can be easily over-stained. Accurate determination of the end point of each step relies largely on practical experience and careful observation of the process.

Over-destaining—In an over-destained specimen, the nerve branches, especially the finer twigs, look faded. Longer destaining periods result in loss of fine detail owing to excessive loss of stain from fine terminal nerve branches. If this occurs, the specimen must be washed thoroughly with running tap water and re-stained in Sihler solution II, then the destaining step repeated.

Checking and dissecting the specimen—During the staining process, the specimen must be checked to determine the appropriate end point for each step. According to our experience, the ideal time for microdissection of a Sihler's stained specimen is 1–2 months after its preservation in pure glycerin. When a specimen is being checked or microdissected, it must be transilluminated with a variable fiberoptic light source; individual nerve branches within the transilluminated specimen can be visualized and traced. Microdissection with a regular dissecting microscope fails to achieve this goal and may damage the nerves and tissues. During dissection, some connective and muscular tissues may be cut off to illustrate all of the intramuscular nerve branches. The main trunk of a given nerve must be identified and followed extramuscularly to its entry point, then traced intramuscularly from major nerve branches to

terminals. Great care should be taken to avoid damage to any noticeable nerve branches and terminal twigs within the tissue. Finally, the well-dissected specimen also should be transilluminated for photographing.

Sample handling—During microdissection of the stained specimens, the dissector may spend much time removing air bubbles within the specimen while tracing intramuscular nerve branches. The air bubbles usually are created by several steps during the staining process. First, they may be produced while the specimen is being washed under running tap water between staining steps. Covering the container with several layers of gauze helps prevent water from falling directly onto the specimen. Second, the specimen should be handled gently when it is squeezed to eliminate excess liquid between staining steps. Finally, air bubbles most likely are produced as glycerin is poured into the container. To avoid this, the container should be tilted 45° to allow the glycerin to flow from the side wall of the container to its bottom. The specimen then is gently placed into the glycerin.

Comparing strengths and limitations

At present, a number of methods allow one to investigate innervation of mammalian skeletal muscles. These include cadaver dissection (Sekiya et al. 1994, Ducic et al. 2006), three-dimensional reconstruction of histological sections (Ohmichi et al. 1988, Loh et al. 2003, Gulekon et al. 2007), Sihler's whole mount nerve staining (Wu and Sanders 1992, Liu et al. 1997), and electrophysiological means, i.e., nerve stimulation, nerve transaction, and electromyography (Furusawa et al. 1991, Kogo et al. 1996, Hammond et al. 1997). It should be noted, however, that each of these methods has its strengths and limitations.

Gross or microdissection with formalin fixed or fresh cadaver specimens is a common method for investigating the innervation of the limb (Sekiya et al. 1994, Ducic et al. 2006) and cranial muscles (Maranillo et al. 2003a,b, 2005, Prades et al. 2006, Yalcin et al. 2006). Unfortunately, direct dissection cannot be used to trace all the intramuscular nerve branches from their main trunks to the terminals. It is difficult to distinguish small nerve branches from blood vessels and connective tissue, and to trace a given nerve branch for a long distance. When tracing intramuscular nerve branches, preserving one branch often necessitates unwanted damage to others (Homma and Sakai 1991). In addition, it is impossible using anatomical dissection to trace individual nerve branches in some complex organs such as the tongue, pharynx, and larynx where the intramuscular nerve branches are very fine and organized into a dense plexus as demonstrated by Sihler's stain (Sanders et al. 1993a,b, Mu and Sanders 1996, 1999, 2007, Ryan et al. 2003).

Three-dimensional reconstruction of a structure or organ can be achieved by tracing images from serial histological or anatomical sections using a computer image processing technique. The major advantage of this technique is that it permits detailed observations of the relations among structures within an organ. Precise three-dimensional modeling and visualization of human anatomy and pathology play an important role in medicine. A good three-dimensional visualization of a given structure or organ is helpful for medical students and residents in their training, and for clinicians in their diagnostic and surgical procedures. Unfortunately, there are few studies directed toward determining intramuscular nerve distribution using this technique (Ohmichi et al. 1988, Loh et al. 2003, Gulekon et al. 2007). It should be noted that computer reconstruction of serial sections is not always accurate owing to distortion during tissue cutting, staining, orientation, and reconstruction. In addition, well-trained technicians are needed for image reconstruction.

Sihler's stain has more advantages than other presently existing methods. This technique has provided valuable information regarding the motor nerve supply patterns in muscles and sensory nerve distribution in the mucosa and skin.

Advantages of Sihler's stain

Entire nerve supply can be mapped without interruption—Unlike direct dissection, which frequently results in unexpected damage to intramuscular nerves and fails to trace small nerve branches, Sihler's stain can render muscle and skin tissues translucent while staining its nerve supply. This technique preserves the integrity of the nerve branches including dense nerve plexuses within a specimen (Fig. 1). Entire nerve mapping of an organ or tissue demonstrates many unknown anatomical facts and clarifies controversies regarding innervation.

Precise intramuscular nerve branching and distribution patterns can be documented—In Sihler's stained specimen, the nerves are stained dark blue or purple and the intramuscular branching and distribution can be seen clearly. A well prepared and stained specimen can demonstrate not only the peripheral course, extramuscular branching, and entry point (motor point) of a given nerve, but also the intramuscular branching and distribution patterns of the nerve branches. The number and diameter of the primary nerve branches also can be determined (Fig. 1). Advanced knowledge of precise intramuscular nerve branching and distribution is especially useful for designing various neurosurgical procedures.

The three-dimensional structure of a whole organ or specimen can be preserved—Sihler's stain preserves the three-dimensional structure of the whole specimen without distorting tissue morphology. Therefore, topographical and morphological relations among the nerve branches, relatively larger blood vessels, muscle fascicles and other structures can be demonstrated (Figs. 1–3). Visualization of the three-dimensional architecture of an organ or tissue is helpful not only for teaching purposes, but also for clinical applications.

Neural organization within the structurally complex organs can be demonstrated—Determination of the nerve supply pattern of a structurally complex organ or structure, especially one innervated by two or more nerves, poses unique challenges because of the complexity of its neuromuscular organization. It is impossible for direct dissection to depict the intricate branching pattern of the nerves and their relations. Sihler's stain is the only technique available for demonstrating the precise distribution patterns of different nerves within such organs as pharynx (Fig. 1) or tongue (Fig. 3). These organs are innervated by multiple nerves and countless terminal branches that form a complex nerve plexus. Details about the innervation patterns of these structurally complex organs are essential for better understanding of their multiple functions.

Neuromuscular compartments (NMCs) within a muscle can be delineated—Sihler's stain is superior to other anatomical methods for determining whether a given muscle is composed of distinct NMCs and, if so, how the NMCs are organized. Sihler's stain has revealed that the NMCs in skeletal muscles can be arranged in parallel (Fig. 4), in series (Fig. 5), or in layers (Fig. 6). Precise delineation of the NMCs within a given muscle is critical for determining their functions and for developing NMC-based therapeutic techniques to treat certain neuromuscular disorders.

Communications between nerves or nerve branches can be visualized—The numbers, types, and locations of connections between nerve branches of a given nerve or between different nerves can be visualized within Sihler's stained specimens (Fig. 7).

Identification of communicating nerve branches facilitates further determination of their functional significance.

Bilaterally innervated muscles or mucosa can be determined—The issue of bilateral innervation of some structures in the body has long been controversial. Some organs, muscles, muscle regions, and mucosa that are located in the midline of the body may receive their innervation from both sides. Sihler's stain has demonstrated that some midline muscles and mucosa in the upper airway are innervated bilaterally. These structures include the interarytenoid muscle in humans (Fig. 8), the rostral compartment within the geniohyoid muscle in dogs (Fig. 9) and the mucosa covering the epiglottis in humans (Fig. 10). Determination of bilateral innervation of these structures has important physiological and clinical implications.

Over-destained and/or poorly stained specimens can be re-stained—Sihler's stained specimens may become faded or opaque after long periods in preservative glycerin. The faded or over-destained specimens can be revitalized by re-staining them in Sihler's solution II followed by destaining and neutralization steps.

Relatively old formalin fixed specimens can be useful for Sihler's stain—Sihler's stain can be used for fresh or old postmortem specimens. While fresh tissue is better than old, specimens from autopsies conducted several days after death also can be stained using this procedure. Sihler's stain also is useful for specimens obtained from cadavers that have been frozen or fixed in formalin for several years.

Stained specimens can be preserved in pure glycerin for many years—Some Sihler's stained specimens in our laboratory have been preserved in pure glycerin for more than 10 years. Intramuscular nerve branches are still visualized clearly even though some terminal branches are faded.

Limitations of Sihler's stain

In spite of the fact that Sihler's stain has many advantages over other anatomical methods, it also has some limitations as described below.

The nature of the nerve branches within a muscle cannot be determined—Because all the nerves in an organ or muscle are stained by the same color, it is impossible to differentiate motor nerves from sensory nerves at the peripheral level. Therefore, each nerve supplying an organ or muscle must be traced from its main trunk to the terminals. In some muscles, communicating nerves between motor and sensory nerves exist (see below). In these cases, the components of the communicating nerve branches cannot be determined by Sihler's stain.

The relations between the nerve terminals and the effector organs cannot be observed in detail—Sihler's technique provides less detailed information concerning the relations of nerve terminal structures. For example, the connections between the motor axons and motor end plates cannot be observed in the stained muscles. The terminal ends of the intramuscular nerve fibers usually are stained weakly. This largely is because repeated branching of a nerve within a muscle causes the intramuscular nerve branches to become thinner. The myelin sheath surrounding the nerve fibers gradually disappears near the nerve terminals (Stevens and Lowe 1997) and the nerve terminals might be too small to take up the stain. Some investigators reported that hematoxylin stains myelinated nerve fibers well, whereas it stains unmyelinated nerve fibers poorly (Liem and Van Willigen 1988). Our studies using Sihler's stain showed that the sensory nerve terminals supplying the epithelium of

laryngeal and pharyngeal mucosa are barely visualized. In Sihler's stained dorsal tongue mucosa, however, the terminals of the lingual (Fig. 11) and glossopharyngeal nerves (Fig. 12) can be seen terminating in the taste buds.

The success of the staining depends on the size of the specimen—In general, the smaller and thinner the muscle, the better the nerve is demonstrated by Sihler's stain. As the size of the muscle increases, clearing of the stained specimen tends to be inadequate; thus, the intramuscular nerve branching and distribution may be shown incompletely. The nerve supply patterns of large muscles, such as human biceps brachii (Fig. 13) can be determined, however, if each of the staining steps is observed carefully.

Sihler's stain is a time-consuming process—The staining process requires several weeks or months, whereas other methods, such as direct dissection or three-dimensional reconstruction, may be accomplished in a shorter time.

Clearly, Sihler's stain has more advantages than disadvantages. Recently, Gulekon et al. (2007) compared anatomical microdissection, Sihler's staining and computerized reconstruction methods for demonstrating intramuscular nerve distribution patterns. Anatomical dissection is a simple method, but it is difficult to demonstrate intramuscular nerve branching and neural connections. By contrast, Sihler's stain and three-dimensional reconstruction can show the intramuscular nerves, but they are complex methods that require long times to complete.

Applications of Sihler's stain

Research groups and their directions

A thorough review of the English literature reveals that 48 articles on the application of the Sihler's stain have been published since it was modified by Liem and Van Willigen (1988). Analysis of the distribution of these articles indicates that Sihler's stain has been employed mainly by several research groups in the USA, Singapore, and Turkey.

In the USA, a research group from the Department of Otolaryngology, Mount Sinai School of Medicine in New York used the modified Sihler's stain in the early 1990s and reported a series of studies (23/48, 48% of the total publications on Sihler's stain). This group made a great effort to determine the intramuscular branching and distribution patterns of the cranial nerves supplying the muscles and mucosa. Wu and Sanders (1992, 1994) were the first to apply the modified Sihler's staining technique to investigate the supply patterns of the laryngeal nerves. Using Sihler's stain, this group investigated extensively innervation of the larynx (Diamond et al. 1992, Drake et al. 1993, Sanders et al. 1993a,b, [1994], Mu et al. 1994, Wu et al. 1994, Sanders and Mu 1998, Mu and Sanders 2009), pharynx (Mu and Sanders 1996, 1998a, 2000a, 2001, 2007, 2008), tongue (Mu and Sanders 1999, 2000b, Zur et al. 2004), and other head and neck muscles (Mu and Sanders 1998b, Ren and Mu 2005) in humans and animals. Other researchers in this country also used Sihler's stain to investigate the nerve supply patterns of the skin (Hirigoyen et al. 1996) and tongue (McClung and Goldberg 2000) in rats; temporalis (Ziccardi et al. 1998), biceps brachii (Amirali et al. 2007) muscles and tongue mucosa (Doty et al. 2009) in humans, and laryngeal musculature in horses (Cheetham et al. 2008). A group from the Departments of Orthopaedic Surgery and Hand and Reconstructive Microsurgery, National University of Singapore, employed Sihler's technique in the late 1990s. This group focused primarily on demonstrating the intramuscular nerve supply patterns of various limb and trunk muscles. Liu et al. (1997) were the first to apply the Sihler's stain to determining intramuscular nerve supply of some limb muscles (long head of triceps) in rabbits. This group further studied innervation of limb and trunk muscles in humans and monkeys (Kumar et al. 1998, Hua et al. 1999, Lim et al. 1999, 2004, Wong et al. 2007). In Turkey, a group from the Department of

Anatomy, Gazi University Faculty of Medicine, in Besevler-Ankara, used Sihler's stain in the early 2000s. Peker et al. (2001, 2003) were the first to investigate the innervation of the masticatory muscles in rabbits. This group also studied the intramuscular distribution of the nerves innervating the diaphragm and extraocular muscles in rabbits (Gozil et al. 2002, Gulekon et al. 2002, Turgut et al. 2006), trunk muscles in rats (Calguner et al. 2006), and limb and trunk muscles from the human fetal cadavers (Peker et al. 2006, Gulekon et al. 2007). Using Sihler's stained human fetal specimens, Peker et al. (2006) investigated the relations between the shape of skeletal muscles and their nerve distribution patterns.

Recently, Sihler's stain has been used by investigators in several other countries to study the innervation of mammalian skeletal muscles (Berkowitz et al. 1997, Kierner et al. 2001, Ryan et al. 2003, Graziotti et al. 2004, Knight et al. 2005).

Species and tissues studied

During the past two decades, Sihler's stain has been employed for human and several animal species including monkeys, horses, dogs, pigs, rabbits, and rats. Overall, studies of the cranial muscles are much more numerous than those of the limb and trunk muscles.

Human studies account for 53% of the total reports on Sihler's technique. Sihler's stained organs, muscles, and other tissues include the larynx (Sanders et al. 1993a,b, 1994, Mu et al. 1994, Wu and Sanders 1994, Wu et al. 1994, Mu and Sanders 2009), pharynx (Mu and Sanders 1996, 1998a, 2001, 2007), masticatory muscles (Ziccardi et al. 1998, Ren and Mu 2005), and adult (Kumar et al. 1998, Hua et al. 1999, Lim et al. 1999, 2004, Kierner et al. 2001, Amirali et al. 2007, Wong et al. 2007) and fetal (Peker et al. 2006, Gulekon et al. 2007) limb and trunk muscles. In addition to motor innervation of skeletal muscles, the sensory nerve supply to the mucosa of the human larynx (Sanders and Mu 1998), pharynx (Mu and Sanders 2000a), and tongue (Zur et al. 2004, Doty et al. 2009) also has been studied using Sihler's stain.

Animal studies account for 47% of the total reports on Sihler's nerve staining. Nerve supply patterns of skeletal muscles and other tissues from the following animal species have been investigated using Sihler's stain. Monkey limb muscles have been investigated using Sihler's stain (Kumar et al. 1998, Hua et al. 1999, Lim et al. 1999). Equine dorsal cricoarytenoid muscle also has been examined using Sihler's nerve staining technique (Cheetham et al. 2008).

Canine organs and muscles studied with Sihler's stain include larynx (Wu and Sanders 1992, Diamond et al. 1992, Drake et al. 1993), suprahyoid muscles (Mu and Sanders 1998b), and tongue (Mu and Sanders 1999, 2000b). Pig laryngeal muscles have been investigated using Sihler's stain (Knight et al. 2005). Rabbit muscles studied using Sihler's stain include limb (Liu et al. 1997), masticatory (Peker et al. 2001, 2003), extraocular (Gozil et al. 2002, Gulekon et al. 2002), laryngeal (Ryan et al. 2003), and diaphragm (Turgut et al. 2006) muscles. Rat organs, muscles, and other tissues investigated using Sihler's nerve staining technique include the hard palate (Liem and Van Willigen 1988), posterior cricoarytenoid muscle (Berkowitz et al. 1997), tongue (McClung and Goldberg 2000), trunk muscles (Calguner et al. 2006), and skin (Hirigoyen et al. 1996).

Contributions of Sihler's stain to neuroscience

Owing to the modified Sihler's nerve staining technique, much progress has been made over the past two decades in our understanding of the innervation of skeletal muscles and mucosa in mammals. The major contributions of Sihler's technique to neuroscience can be summarized as new discoveries, clarification of controversies, and others as described below.

Sihler's stain-related discoveries

Sihler's stain has enabled a number of new discoveries that greatly improve our knowledge of neuromuscular systems. For example, as generally described, the pharyngeal constrictor muscles receive their motor innervation from the pharyngeal plexus. Recently, Mu and Sanders (2007) investigated the pharyngeal constrictors using Sihler's stain and other techniques. Immunocytochemical studies showed that human pharyngeal constrictors consist of two layers, a slow inner layer (SIL) and a fast outer layer (FOL). Sihler's stain demonstrated that the SIL is innervated by the glossopharyngeal nerve (IX), whereas the FOL is supplied by the vagus (X) nerve. The cranial nerve IX branch innervating the SIL was confirmed to contain motor axons. These findings may be contrary to the now-prevailing view that cranial nerve IX only provides motor innervation to the stylopharyngeus muscle, while cranial nerve X innervates all of the pharyngeal constrictors (Standring et al. 2005). On the basis of their new findings, Mu and Sanders (2007) propose that the motor tasks that occur in the human pharynx are controlled by two neuromuscular sets, the SIL of cranial nerve IX and the FOL of cranial nerve X.

More recently, Mu and Sanders (2008) described a new muscle termed “cricothyropharyngeus” in the human laryngopharynx. The cricothyropharyngeus originates from the anterior arch of the cricoid cartilage, and courses between the inferior pharyngeal constrictor and cricopharyngeus muscles to insert into the median raphe at the posterior midline of the pharynx. Sihler's stain showed that the innervation of the cricothyropharyngeus differs from that of neighboring muscles. Specifically, the cricothyropharyngeus receives its innervation from two different sources, one from the external superior laryngeal nerve and another from the pharyngeal plexus. Importantly, this new muscle is not found in commonly used experimental animals, such as dogs, pigs, rabbits, and rats. The authors believed that the cricothyropharyngeus is a uniquely human muscle with characteristics suggesting a specialized function that may be related to speech.

Clarification of some controversies concerning muscle innervation

There are many controversies and uncertainties concerning the innervation of some organs, skeletal muscles, and other tissues. Studies have demonstrated that many traditional descriptions of innervation of skeletal muscles are inaccurate. This is due largely to the fact that most of the previous studies were based on gross dissection and/or electrophysiological analysis. For example, the formation and distribution of the nerves forming the pharyngeal plexus have been controversial for more than a century (Mu and Sanders 1996, Sasaki 2000, Lang 2006 for reviews). Mu and Sanders (1996, 1998a, 2000a, [2001], 2007) were the first to apply Sihler's nerve staining to the human pharynx and to map the entire pharyngeal plexus. Therefore, the branching and distribution of the motor and sensory nerves supplying the pharynx have been determined. The data obtained from Sihler's stained specimens are very helpful for clarifying certain controversies and uncovering some previously unknown anatomical facts about this organ.

Another important issue is the distribution of cranial nerve IX in the tongue. Nineteenth century anatomical descriptions of the distribution of cranial nerve IX on the anterior dorsal tongue are contrary to current views. It is generally believed today that the distribution of cranial nerve IX is limited to the posterior third of the tongue. Doty et al. (2009) were the first to use Sihler's stain to clarify this controversial issue. They demonstrated that cranial nerve IX projects more anteriorly than the traditionally defined posterior third of the tongue. These findings are critical for determining further the role played by cranial nerve IX in taste function anterior to the circumvallate and foliate papillae.

There also are controversies regarding the motor innervation of some limb and trunk muscles. For example, the spinal accessory nerve, cranial nerve XI, generally is regarded as the sole motor innervation to the trapezius muscle (Standring et al. 2005). Various sources of nerve supply to this muscle have been described, however, and data available on this subject are confusing and even contradictory. Clinicians, anatomists, and physiologists have made great efforts to determine the accurate innervation of the trapezius muscle, because loss of function in the shoulder girdle is common in patients after radical neck dissection. Unfortunately, there is still considerable disagreement between anatomists and surgeons regarding the motor innervation of the trapezius muscle. Most anatomists consider the spinal accessory nerve to be the only motor nerve supply to the trapezius muscle, whereas from the physicians' point of view, both the spinal accessory nerve and the cervical plexus branches are mixed nerves, contributing more or less equally to the motor innervation of the muscle (Kierner et al. 2000, 2001 for review). Kierner et al. (2001) were the first to employ Sihler's technique for clarifying this important issue. They found that descending part of the trapezius muscle in humans is supplied by a branch from the spinal accessory nerve, whereas the transverse and ascending parts are innervated by both the spinal accessory nerve and the trapezius branches of the cervical plexus.

Precise delineation of neuromuscular compartments (NMCs) within a muscle

There is a growing body of evidence that supports the concept of NMCs proposed by English and Letbetter (1982a,b) and Windhorst et al. (1989). Many skeletal muscles thought to be single muscles actually are composed of morphologically and functionally distinct NMCs that work independently during different physiological tasks (Bodine et al. 1982, English and Weeks 1987, Chanaud et al. 1991, Zaretsky and Sanders 1992). The functional NMCs within a muscle are always delineated first by anatomical and histochemical criteria, such as distinct muscle fiber arrangement, separate nerve supply, and fiber type regionalization. Among these parameters, separate motor nerve supply to distinct muscle regions strongly indicates the presence of NMCs (English and Letbetter 1982a, Windhorst et al. 1989, English et al. 1993). Precise intramuscular innervation pattern of a muscle, however, cannot be demonstrated reliably by gross dissection.

Sihler's stain allows one to visualize all the nerve branches and their distribution within a given muscle, thus reliably determining whether or not the muscle is compartmentalized. Sanders et al. (1993b, 1994) were the first to demonstrate that most of the laryngeal muscles are composed of two or more NMCs as revealed by Sihler's stain. Subsequent studies by Mu and Sanders (1998a,[b], 2000b, 2001, 2007) showed that the majority of the muscles of the mouth and pharynx also are compartmentalized. More recently, Mu and Sanders (2009) presented evidence showing that the human cricothyroid muscle was composed of three bellies, two traditionally described (rectus and oblique) (Standring et al. 2005) and a newly revealed horizontal belly, each innervated by separate branches of the external superior laryngeal nerve. In addition to the muscles mentioned above, NMCs within some limb muscles also have been delineated using Sihler's stain. Liu et al. (1997) were the first to document that the long head of the triceps muscle in the rabbit is composed of three NMCs. Subsequently, NMCs have been delineated within the gracilis (Kumar et al. 1998), flexor carpi radialis (Hua et al. 1999), and flexor carpi ulnaris (Lim et al. 1999) muscles in humans and monkeys.

The NMCs in the skeletal muscles can be arranged in parallel (Sanders et al. 1994, Mu and Sanders 1998b, 2000b, 2009), in series (Bodine et al. 1982, English and Weeks 1987, Mu and Sanders 1998b), or in layers (Mu and Sanders 2001, 2002, 2007, Mu et al. 2007a,b), possibly depending on the shapes and functional demands of the muscles. The NMCs within a muscle may be supplied by the primary nerve branches derived from the same nerve (Sanders et al. 1994, Mu and Sanders 1998b, 2000b, 2009) or from different nerves (Kierner et al. 2001, Mu

and Sanders 2007). Unfortunately, the intramuscular nerve supply patterns of most skeletal muscles remain to be determined. To this end, Sihler's stain is superior to other anatomical methods. Fiber type regionalization has been found in compartmentalized muscles (Bodine et al. 1982, English and Letbetter 1982b, Chanaud et al. 1991, Zaretsky and Sanders 1992, Korfage and Van Eijden 1999, Mu and Sanders 2001, 2002, 2007, Mu et al. 2007a,b) and demonstrated to be in accord with regional differences in muscle function (Bodine et al. 1982, Chanaud et al. 1991, Zaretsky and Sanders 1992). The muscles or NMCs with a high proportion of type I fatigue-resistant fibers generally are involved in sustained contraction and postural adjustments, whereas those with a high proportion of fast type II fibers are associated with phasic and rapid movements (Hoh 1992). Taken together, NMCs within a muscle can be defined reliably anatomically and histochemically. Separate nerve supply patterns of a muscle strongly indicate that the muscle is compartmentalized.

Reliable identification of the neural connections between branches of different nerves

Neural communications in a given organ play an important role in transporting messages delivered by the central nervous system. Therefore, identifying and localizing the neural connections between nerves or nerve branches is helpful for better understanding and for exploring their functions. Gross dissection may identify some neural anastomoses between large nerve branches (Dilworth 1921, Vogel 1952, Durham and Harrison 1964, Sanudo et al. 1999). This approach has been limited, however, by the inability to trace the individual intramuscular nerve branches and to identify precisely the intramuscular neural connections, especially those between small nerve branches. By contrast, Sihler's stained specimens allow reliable identification of various neural connections between different nerves or branches supplying cranial (Sanders et al. 1993b, Mu et al. 1994, Wu et al. 1994, Mu and Sanders 1999, 2009, Peker et al. 2001, Ryan et al. 2003, Zur et al. 2004) and limb (Liu et al. 1997) muscles, and the mucosa of the larynx and pharynx (Sanders and Mu 1998, Mu and Sanders 2000a). Importantly, Silher's stain can show whether the neural communications occur between branches of the same nerve (Liu et al. 1997, Peker et al. 2001, Ryan et al. 2003) or between different nerves (Mu et al. 1994, Wu et al. 1994, Mu and Sanders 1999, 2009, Zur et al. 2004).

The frequency and constancy of neural connections within a given muscle or tissue may vary with species, individuals, and organs or tissues studied. For example, the external superior laryngeal nerve–recurrent laryngeal nerve connections have been identified in 6–68% of the specimens examined (Dilworth 1921, Durham and Harrison 1964, Wu et al. 1994, Sanudo et al. 1999, Maranillo et al. 2003b, Mu and Sanders 2009), whereas the external superior laryngeal nerve–internal superior laryngeal nerve connections have been found in 4–30% of the total dissections (Dilworth 1921, Vogel 1952, Durham and Harrison 1964, Wu et al. 1994, Sanudo et al. 1999, Maranillo et al. 2003b). On the other hand, the wide range of reported percentages of neural connections between two nerves or nerve branches appears to be associated at least in part with the methods used. It should be noted that it is very difficult for direct dissection to distinguish a fine nerve branch from a small blood vessel or strip of connective tissue that may be interpreted incorrectly as a communicating nerve. By contrast, Sihler's stain has no such drawbacks.

Although neural communications may be found in any skeletal muscles, mucosa or other tissues, and exist constantly or occasionally, the following types of the anastomotic connections usually are found and may be regarded as having significant physiological and clinical implications.

Motor–motor connection between two branches from a single nerve—This type of neural communication refers to the connections between two motor nerve branches that are

derived from the same nerve, but supply two distinct regions or compartments within a muscle or two different muscles. For example, neural connections have been found between two primary branches from the external superior laryngeal nerve that innervate rectus and oblique bellies of the cricothyroid muscle (Fig. 14) (Mu and Sanders 2009) and between the recurrent laryngeal nerve branches supplying the posterior cricoarytenoid and interarytenoid muscles (Fig. 15) (Sanders et al. 1994,Ryan et al. 2003). This type of neural connection appears to be common in limb muscles (Liu et al. 1997). Clinically, if one of the two motor nerve branches is transected proximal to the neural connection, the function of the affected compartment or muscle may be retained partially by intact motor axons from another nerve branch through the neural communications.

Motor–motor connection between two different motor nerves—This type of neural communication refers to the connections between two branches derived from two different motor nerves that may innervate the same region or different regions within a given muscle, or supply different muscles. For example, neural connections between the recurrent laryngeal nerve and pharyngeal plexus in the cricopharyngeus muscle (Fig. 16) (Mu and Sanders 1996) and between the external superior laryngeal nerve and recurrent laryngeal nerve (Fig. 17) (Wu et al. 1994,Mu and Sanders 2009) have been identified using Sihler's nerve staining. Clinically, if one of the motor nerves is transected proximal to the neural connection, partial function of the affected muscle may be retained by innervation from another nerve via the neural connections.

Motor–sensory or sensory–motor connection between motor and sensory nerves—This type of neural communication refers to the connections between a motor nerve and a sensory nerve. For example, neural connections have been identified between the recurrent laryngeal nerve and the internal superior laryngeal nerve in the interarytenoid muscle (Fig. 18) (Mu et al. 1994), between the external superior and internal superior laryngeal nerves in the larynx (Wu et al. 1994), and between the lingual nerve and hypoglossal nerve (XII) in the tongue (Fig. 19) (Mu and Sanders 1994,Zur et al. 2004). It generally is accepted that the recurrent laryngeal nerve, external superior laryngeal nerve, and cranial nerve XII are motor, whereas the internal superior laryngeal and lingual nerves are sensory. It should be pointed out that the nature of the internal superior laryngeal nerve supplying the interarytenoid muscle must be clarified further, because some investigators believe they may contain motor axons (Mu et al. 1994). Because the composition and course of the axons in the communicating nerve branches remain unknown, it would be a motor–sensory connection if the recurrent laryngeal–internal superior laryngeal nerve communicating branch contains only recurrent laryngeal nerve motor axons and the internal superior laryngeal nerve is a purely sensory nerve. By contrast, it is a sensory–motor connection if the recurrent laryngeal–internal superior laryngeal nerve communicating branch contains only the internal superior laryngeal nerve sensory fibers. This would be the case also for the lingual nerve–cranial nerve XII connections.

Sensory–sensory connection between two nerve branches derived from a single nerve—This type of neural communication refers to connections between two sensory nerve branches that are derived from the same nerve, but supply two distinct regions of mucosa within an organ. For example, neural connections between two primary nerve branches from the cranial nerve-IX in the dorsal mucosa of the posterior tongue (Fig. 20) have been found using Sihler's technique (Mu and Sanders 2000a). In this case, if one of the nerve branches is transected proximal to the neural connection, the function of the transected branch may be taken over by another branch via the neural connection.

Sensory–sensory connection between two different sensory nerves—This type of neural communication refers to connections between two branches derived from two

different sensory nerves that may supply the same region or different regions of mucosa within an organ. For example, lingual nerve–cranial nerve IX connections (Fig. 21) in the dorsal mucosa of the posterior tongue (Mu and Sanders 1999,2000a) and cranial nerve IX–internal superior laryngeal nerve connections (Fig. 22) in the mucosa surrounding the epiglottis (Mu and Sanders 2000a) have been identified using Sihler's stain. These findings may explain why the epiglottis retains some degree of sensibility after bilateral section of the internal superior laryngeal and recurrent laryngeal nerves (Feindel 1956).

Reliable visualization of additional and bilateral innervation of muscles and mucosa

It is traditionally claimed that all laryngeal muscles are innervated by the recurrent laryngeal nerve except the cricothyroid muscle, which is innervated by the external superior laryngeal nerve (Standring et al. 2005). Using Sihler's stain, Wu et al. (1994) and Mu and Sanders (2009) demonstrated an additional pathway from the external superior laryngeal nerve to the thyroarytenoid muscle. In addition, pharyngeal constrictor muscles generally are believed to be innervated by the pharyngeal branches of cranial nerve X (Standring et al. 2005). Using Sihler's stain, however, Mu and Sanders (2007) have demonstrated that the human pharyngeal constrictor muscles also receive motor innervation from cranial nerve IX. Evidently, additional innervation sources to a given muscle can be visualized reliably using Sihler's nerve staining technique.

Sihler's stain also has demonstrated that some midline muscles, such as the interarytenoid muscle in the human larynx (Mu et al. 1994) and the rostral compartment of the canine geniohyoid muscle (Mu and Sanders 1998b) are innervated bilaterally. In the laryngeal and pharyngeal mucosa, several dense sensory plexuses formed by the internal superior laryngeal nerve that cross the midline have been observed on the laryngeal surface of the epiglottis and arytenoid region (Sanders and Mu 1998, Mu and Sanders 2000a). There is significant crossover between the bilateral lingual nerves at the anterior tip of the tongue (Zur et al. 2004) and between the bilateral cranial nerves IX at the base of the tongue (Mu and Sanders 2000a). Identification of bilaterally innervated muscles and mucosa is important for clinical evaluation of motor or sensory function and for correct interpretation of nerve injury experiments. For example, transection of one cranial nerve XII in the dog may not completely paralyze the rostral geniohyoid compartment because of bilateral innervation.

Significance of Sihler's technique in physiological and clinical applications

Accurate knowledge of intramuscular innervation patterns of skeletal muscles and mucosa as demonstrated by Sihler's stain is important not only for anatomists, but also for physiologists, muscle researchers, and clinicians to understand further the functions of these structures and to advance diagnosis and treatment of neuromuscular disorders.

Physiological functions of a given muscle can be understood better by knowing its innervation pattern. In general, a compartmentalized muscle is structurally designed for multiple functions. Electrophysiological studies usually are performed to document the functional behaviors of the compartments within a muscle. Details about extra and intramuscular branching patterns of the nerves innervating the compartments are critical for guiding neurophysiological experiments, such as neural recordings, selective nerve stimulation, and various nerve injury models. These experiments cannot be carried out optimally without knowing the precise branching and distribution of the nerve or nerves supplying a muscle. For example, to establish a denervation model, it is important to know whether the muscle tested is innervated ipsilaterally or bilaterally by a single nerve or multiple nerves. The experimenter also should be aware of whether communicating nerve branches are present in the muscle. For example, if the axons from the posterior cricoarytenoid branch supply the interarytenoid muscle via the communicating nerve as described above, the interarytenoid muscle cannot be expected to be

paralyzed completely following transection of the nerve to the interarytenoid muscle proximal to the communicating branch. Taken together, accurate neuroanatomical data are critical for physiological studies.

Diagnosis of neuromuscular disorders requires thorough knowledge of the neuroanatomy. Sihler's stain has demonstrated that the thyroarytenoid muscle receives its motor innervation not only from the traditionally described recurrent laryngeal nerve, but also from the external superior laryngeal nerve (Wu et al. 1994, Mu and Sanders 2009). This finding has important clinical implications for diagnosis and evaluation of laryngeal paralysis. For example, electromyograph (EMG) may show the persistence of some degree of voluntary muscle activity in a vocal fold following recurrent laryngeal nerve transection. A possible explanation for the residual EMG activity could be the additional innervation of the vocal fold provided by the external superior laryngeal nerve through direct nerve supply and/or neural communications between the external superior laryngeal and recurrent laryngeal nerves.

Sihler's stain allows one to demonstrate more accurately and completely the neuroanatomy of the skeletal muscles and this should assist in guiding and/or developing novel reinnervation procedures and a wide range of therapeutic techniques for treating neuromuscular disorders. For example, electrical stimulation of the genioglossus muscle (Schwartz et al. 1996, Smith et al. 1996, Oliven et al. 2003) or hypoglossal nerve (the main trunk or genioglossus branch) (Eisele et al. 1997, Oliven et al. 2003) has been employed to treat patients with obstructive sleep apnea, a life-threatening disease which affects about 20 million Americans (National Institutes of Health 1993, Young et al. 1993). Because the genioglossus muscle is a major tongue protrudor, its diminished activity during sleep leads to prolapse of the tongue base into the pharyngeal airway, which is thought to be the cause of obstructive sleep apnea (Remmers et al. 1978, Wheatley et al. 1993, White 2006). More recently, Durand and colleagues have developed a flat interface nerve electrode for functionally selective peripheral nerve stimulation that is applied to the hypoglossal nerve and others (Tyler and Durand 2002, Yoo and Durand 2005, Durand 2007). The location of the nerve branches and the nerve entry points in a given muscle can be demonstrated by Sihler's stain. This information facilitates placement of electrodes for functional electrical stimulation and can be used to identify efficient and effective sites at which to apply stimulation and to identify stimulation parameters that are likely to produce the desired therapeutic effects.

Many neurosurgical procedures require surgeons to have a sound knowledge of the neuromuscular anatomy. For example, the nerve–muscle pedicle technique has been used to treat a paralyzed larynx (Tucker 1976, 1989, Zheng et al. 1998, Toth et al. 2005) and face (Hall et al. 1988). Precise location of the motor point and the nerve branching pattern are critical for designing a nerve–muscle pedicle.

Another commonly used microsurgical procedure for dynamic smile reconstruction of longstanding facial paralysis is the neurovascular free-muscle transfer. Free flaps for facial reanimation can be obtained from limb and trunk muscles including gracilis (Sassoon et al. 1991, Terzis and Noah 1997, Bae et al. 2006, Faria et al. 2007), latissimus dorsi (Dellon and Mackinnon 1985, Wei et al. 1999, Harrison 2002, Faria et al. 2007), pectoralis minor (Terzis and Noah 1997, Harrison 2002), biceps femoris (Hayashi and Maruyama 2005), and rectus (Marek and Pu 2004) or oblique (Wang et al. 2002) abdominus. This strategy also has considerable potential for treating other skeletal muscle paralyses. Determination of the NMCs within skeletal muscles is helpful for designing compartment-based surgical procedures, such as free-muscle transfer. For example, Sihler's stain has revealed that the gracilis muscle is composed of two or more NMCs that are arranged in parallel (Hua et al. 1999). These findings suggest that the compartments can be split longitudinally for free compartment transfer. A free compartment with its own nerve branch may be used locally as a pedicle or transferred to a

distant site (Hua et al. 1999, Lim et al. 1999). Free muscle transfer or musculocutaneous flaps also are used for head, neck, and extremity reconstruction of a variety of soft tissue defects caused by trauma or tumor resection (Kim et al. 2001, Wei et al. 2002, Zhang et al. 2004) and for obliteration of dead space in infected cavities and fistulous communications (Heckler et al. 1980). The current challenge in reconstructive microsurgery is re-establishment of sensorimotor function. It has been reported that free muscle flaps with sensory to motor coaptation appear to develop sensibility (Ninkovic et al. 1998, Bayramicli et al. 2000, Herter et al. 2007). All of these procedures can be carried out successfully only when the course and distribution pattern of the nerve branches in the skeletal muscle are well defined.

Taken together, it can be expected that Sihler's stain could reveal more neurovascular free muscle or musculocutaneous flaps for reconstruction and may lead to new avenues for innovative neurosurgical techniques.

Future studies using Sihler's stain

Sihler's stain clearly is a powerful and reliable technique for visualizing the branching and distribution patterns of peripheral nerves. It has made great contributions to neuroscience and has advanced our understanding of the functional organization of the peripheral nervous system. Importantly, the data concerning intramuscular branching and distribution of peripheral nerves are valuable for physiological and clinical applications. Further studies are needed, however, for improving this technique and expanding its utilization in a variety of research fields.

Technical improvement

Although Sihler's stain is superior to other anatomical methods for demonstrating intramuscular distribution of peripheral nerves, it has some limitations as mentioned earlier in this review. More work is needed to improve technical aspects of Sihler's stain for optimal staining results.

Clearing larger Sihler's stained organs or muscles presents a difficult challenge. To resolve this problem, researchers may attempt to inject maceration (3% KOH) and destaining (Sihler's I) solutions into the central portion of a large muscle to achieve adequate depigmentation and destaining, respectively.

Another technical issue is the long-term preservation of the stained specimens. In recent years, Peker et al. (2003) introduced a clearing and embedding method using polyester resin for Sihler's stained specimens. The Sihler's stained muscles appear to be cleared more successfully and the nerve distribution is visualized better in polyester than in the more commonly used glycerin. Other approaches may be tried to preserve and exhibit better the stained specimens for teaching and training medical students, clinical residents, and junior surgeons.

One of the limitations of Sihler's stain is that the continuity of the peripheral nerve fibers with the motoneurons in the CNS and motor end plates in muscles is not preserved. These obstacles may be overcome by improving and modifying the staining procedures. Simultaneous visualization of the nerve terminals and motor end plates in one specimen would be helpful for understanding the relations between nerve terminals and motor end plates.

Expansion of research scope

Despite its scientific and clinical importance, Sihler's stain still is used only by research groups in a few countries (Table 1). It may be expected, however, that Sihler's stain will be more widely employed and will gain greater popularity in many research and clinical fields as described below.

Although Sihler's stain has been used increasingly during the last two decades, it has been applied only to a small number of skeletal muscles. In other words, accurate nerve supply patterns of the majority of skeletal muscles of the body remain to be determined using this technique. In the future, we should expand our research scope to demonstrate the nerve supply patterns of skin, mucosa, glands, blood vessels, smooth muscles, and even an entire body. It is possible for Sihler's technique to stain all the peripheral nerves supplying the whole body, at least in small mammalian species such as rodents. A whole body nerve map would have important significance for neuroscience and clinical practice.

In addition to normal tissues as mentioned above, Sihler's stain may be used as a critical tool to detect pathological conditions, such as muscle denervation. This technique would also be useful for evaluating the extent of neuroregeneration following reinnervation of a paralyzed organ or muscle. These possibilities can be determined using Sihler's stain in animal experiments. If it works, this technique could play an important role in assessing functional recovery and the success of neurosurgical procedures.

Simultaneous mapping of the nerves and blood vessels supplying the muscles and skin would have clinical significance. A detailed description of the neurovascular territories in these structures is particularly important for designing flaps for reconstructive surgery. The use of flaps to reconstruct soft tissue defects requires detailed knowledge of the local vasculature. The morphometry and vascular anatomy of skin or a muscle can be determined by cadaver dissection after arterial injection with red latex (Pinar et al. 2005, Bilge et al. 2007). Some investigators (Hua et al. 1999, Lim et al. 1999) studied the nerve supply of a muscle using Sihler's stain, while the vascular anatomy of the same muscle was examined using different cadavers and injection of 10% aqueous barium sulfate and subsequent radiography. Unfortunately, simultaneous mapping of nerves with Sihler's stain and blood vessels with latex injection is lacking. With simultaneous neural and vascular mapping, the three-dimensional architecture of the nerves and blood vessels within a muscle or organ could be visualized in a single specimen.

Sihler's stain also would be expected to permit documentation of the changes in the intramuscular nerve supply pattern during muscle development. Using Sihler's stain, Peker et al. (2006) and Gulekon et al. (2007) investigated nerve supply patterns of human fetal limb and trunk muscles. Similar work should be conducted on many other skeletal muscles to establish a solid data base that would be helpful for better understanding of the development of the peripheral nervous system.

Finally, comparative studies also are helpful for clarifying discrepancies concerning innervation of a given muscle reported in the literature. For example, the inferior pharyngeal constrictor muscle in monkeys, dogs, and cats has been reported to be innervated by the pharyngeal branch of the vagus nerve. By contrast, the human inferior pharyngeal constrictor was found to be supplied by the pharyngeal branch of the vagus, the recurrent and superior laryngeal nerves, or by a combination of the three nerves, even though the investigators used the same method, direct dissection. In addition, different findings were obtained from the same species by different investigators and by using different methods. In dogs, for example, dissection studies showed that the cricopharyngeus muscle was innervated by the pharyngeal branch of the vagus, whereas electrical stimulation experiments reported that it was innervated by the recurrent laryngeal nerve or sympathetic and parasympathetic nerves (Mu and Sanders 1996 for review). The discrepancies concerning innervation of a given muscle reported in the literature may be species differences between humans and experimental animals. Interspecies variations of motor innervation of a given muscle suggest that intramuscular nerve supply patterns may be modified according to the unique functions of the muscle. On the other hand, the reported inconsistent and even contradictory findings may be attributed, at least in part, to

the methods used. Therefore, it is difficult to explain why the investigators who used the same method to study the same muscle in the same species obtained different results and why the results gained from a given muscle in a given species varied with different methods.

Comparative studies using Sihler's stain are crucial for determining species differences in the neural organization of a given muscle. Using Sihler's stain, some investigators have shown a close resemblance between monkey and human concerning intramuscular innervation patterns of the gracilis (Kumar et al. 1998), flexor carpi radialis (Hua et al. 1999), and flexor carpi ulnaris (Lim et al. 1999) muscles. This information is important for selecting an ideal animal model on which to perform electrophysiological experiments and/or neurosurgical procedures.

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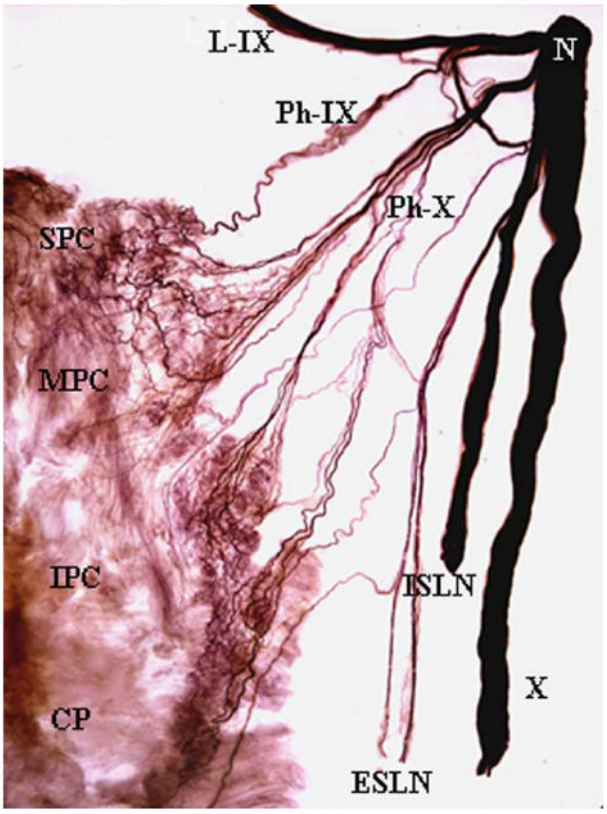

Fig. 1.

Posterior view of a harvested adult human hemipharynx processed with Sihler's stain showing entire pharyngeal plexus. Note that the nerves supplying the pharyngeal muscles were divided at the level of the nodose ganglion (N) of the vagus (X) nerve. Individual nerve branches can be traced from their origins to their terminals. CP, cricopharyngeus muscle; ESLN, external superior laryngeal nerve; IPC, inferior pharyngeal constrictor muscle; ISLN, internal superior laryngeal nerve; L-IX, lingual branch of the glossopharyngeal (IX) nerve; MPC, middle pharyngeal constrictor muscle; Ph-IX, pharyngeal branches of the IX nerve; Ph-X, pharyngeal branches of the X nerve; SPC, superior pharyngeal constrictor muscle. 3 ×.

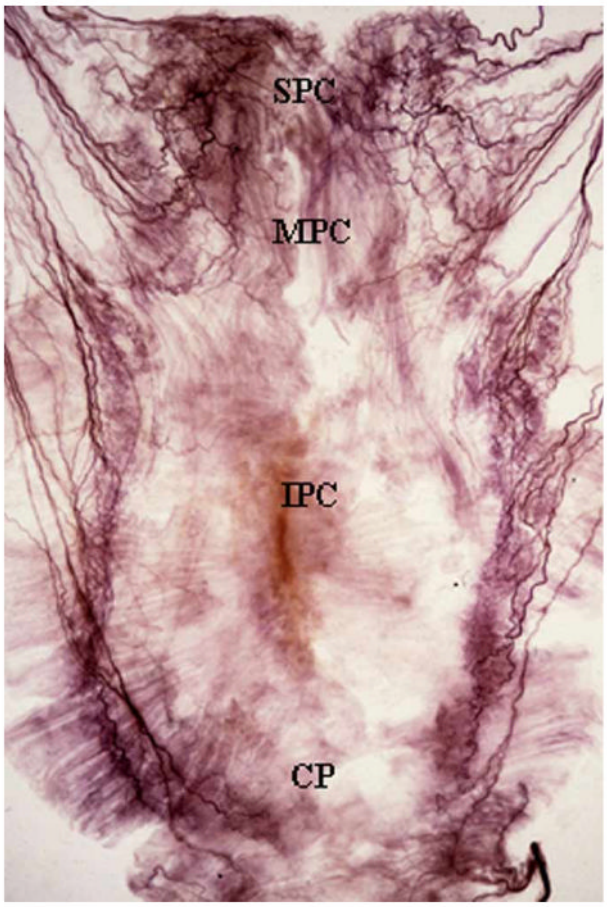

Fig. 2.
Posterior view of a Sihler's stained incompletely harvested adult human pharynx. Note that nerves forming the pharyngeal plexus were not transected at the level of the nodose ganglion of the cranial nerve X. Therefore, it is impossible to know which nerve branches come from the cranial nerve X or from other nerves. The abbreviations used in this figure are the same as those in Fig. 1. 3 ×.

Fig. 3.
Ventral view of the anterior adult human tongue processed for Sihler's stain. Note that the human tongue is supplied by the hypoglossal (XII; motor), lingual (LN; sensory), and glossopharyngeal (IX; sensory) nerves, which are organized in a complex manner. 3 ×.

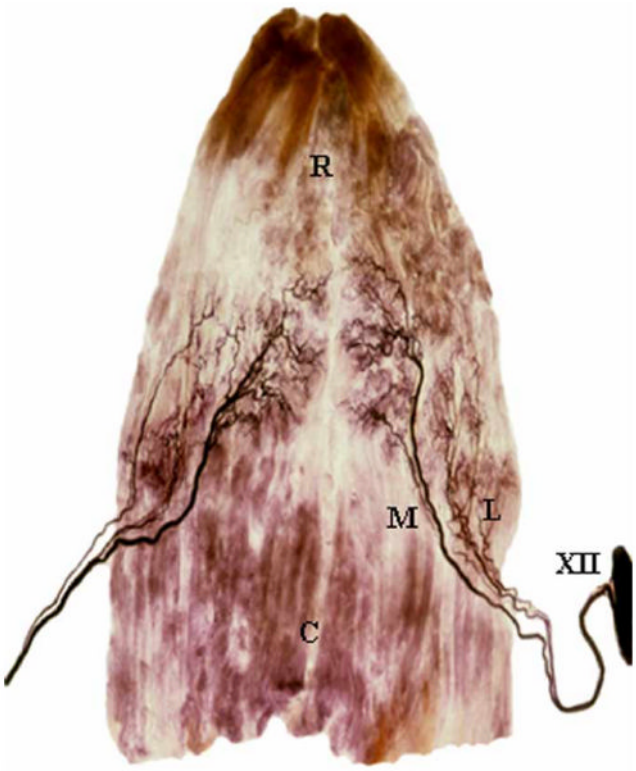

Fig. 4.

A pair of human geniohyoid (GH) muscles processed with Sihler's stain. Note that the human geniohyoid on each side is composed of two neuromuscular compartments, medial (M) and lateral (L), that are arranged in parallel. Note that each of the geniohyoid compartments is supplied by a distinct nerve branch derived from the hypoglossal (XII) nerve. C, caudal; R, rostral. 3 ×.

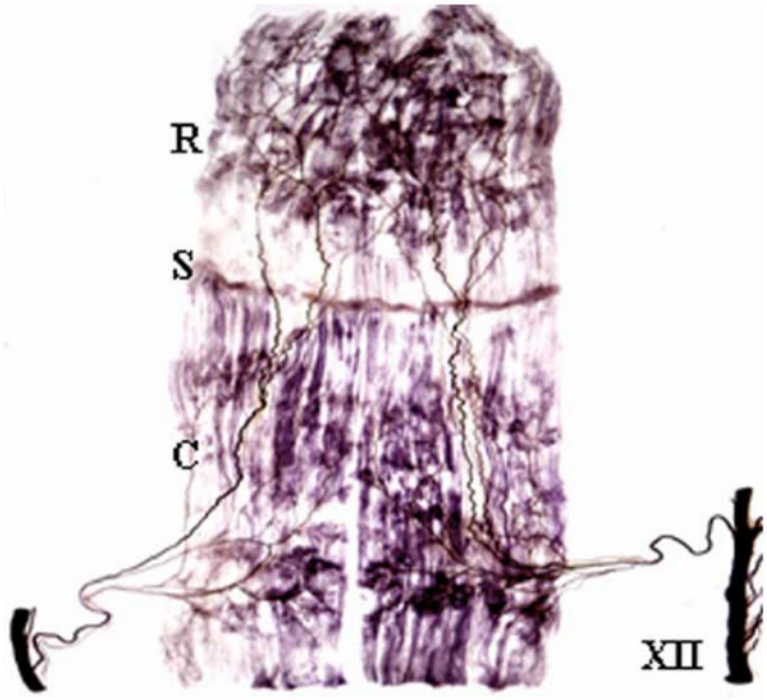

Fig. 5.

A pair of canine geniohyoid (GH) muscles processed with Sihler's stain. Note that the canine geniohyoid is composed of two neuromuscular compartments, rostral (R) and caudal (C) that are separated by a fibrous septum (S) and arranged in series. Note that each of the geniohyoid compartments is supplied by a distinct primary nerve branch derived from the hypoglossal (XII) nerve. 3 ×.

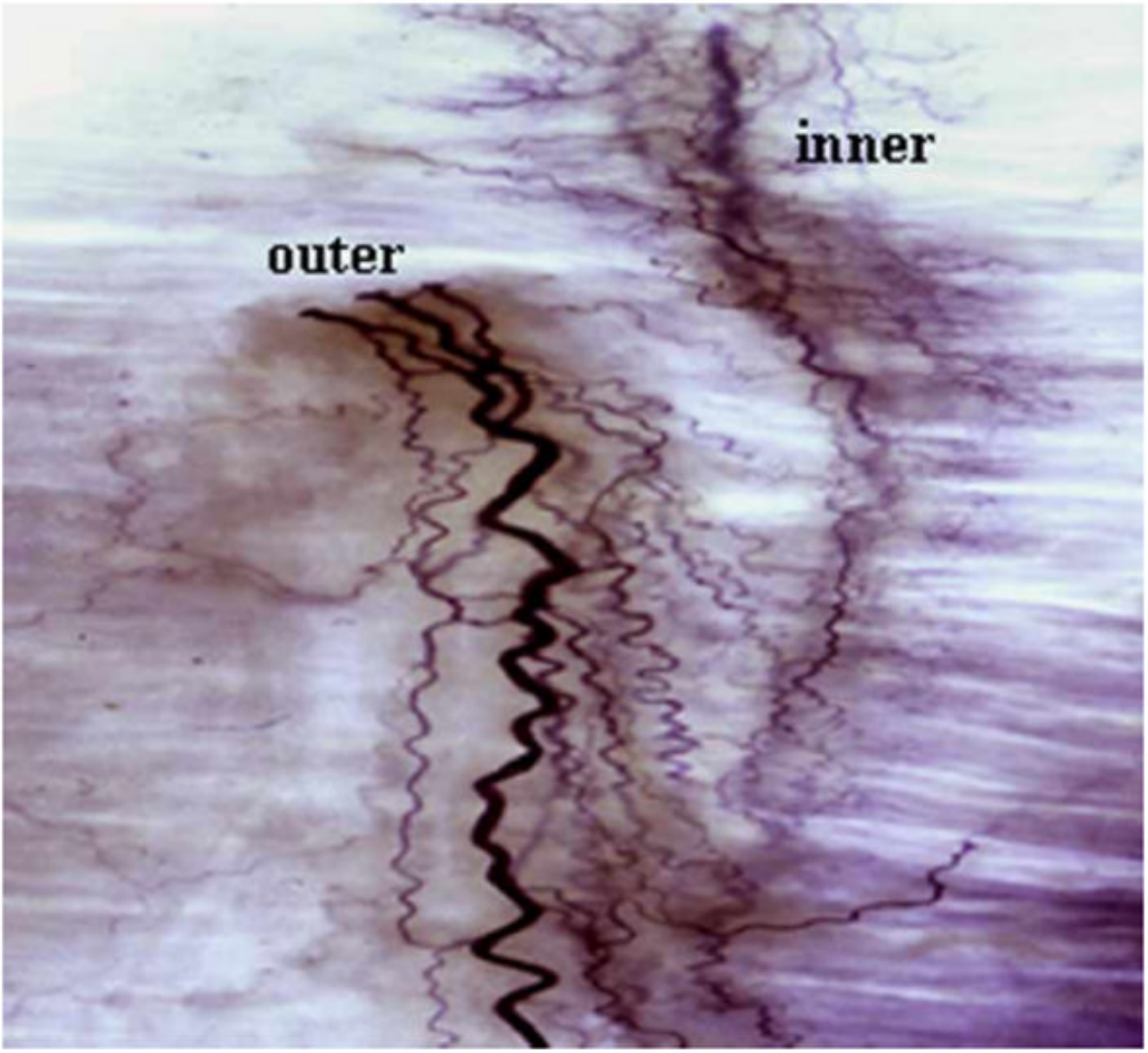

Fig. 6.
Sihler's stained adult human hemipharynx showing that the pharyngeal muscles are composed of neuromuscular compartments arranged in layers, inner and outer. Note that both muscle layers are supplied by different nerve branches from the pharyngeal plexus. 6 ×.

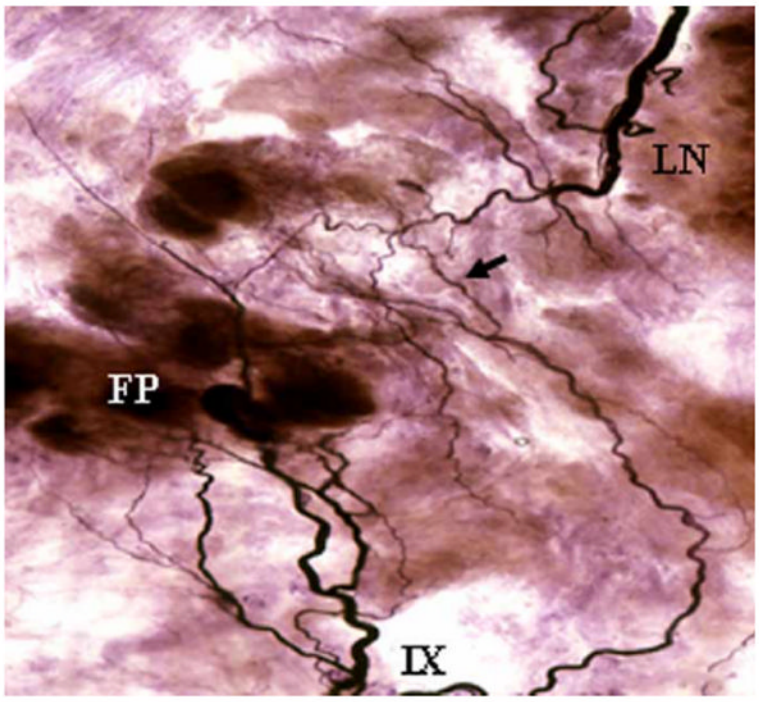

Fig. 7.
Dorsal mucosa of human posterior tongue processed with Sihler's stain showing the neural connection (arrow) between two sensory nerves, the lingual (LN) and glossopharyngeal (IX) nerves. FP, foliate papillae. 12 ×.

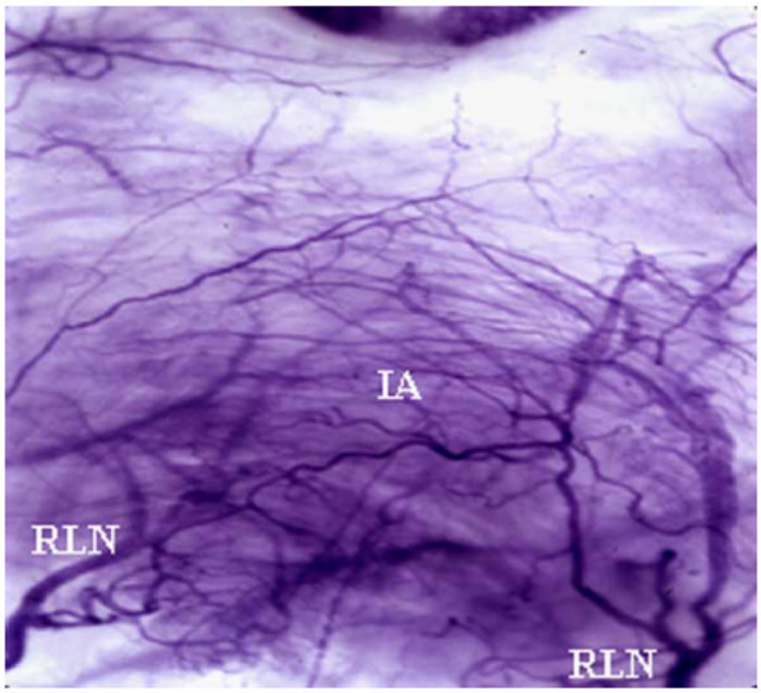

Fig. 8.
Sihler's stained adult human interarytenoid (IA) muscle. Note that the interarytenoid muscle is innervated bilaterally by recurrent laryngeal nerve (RLN). 9 ×.

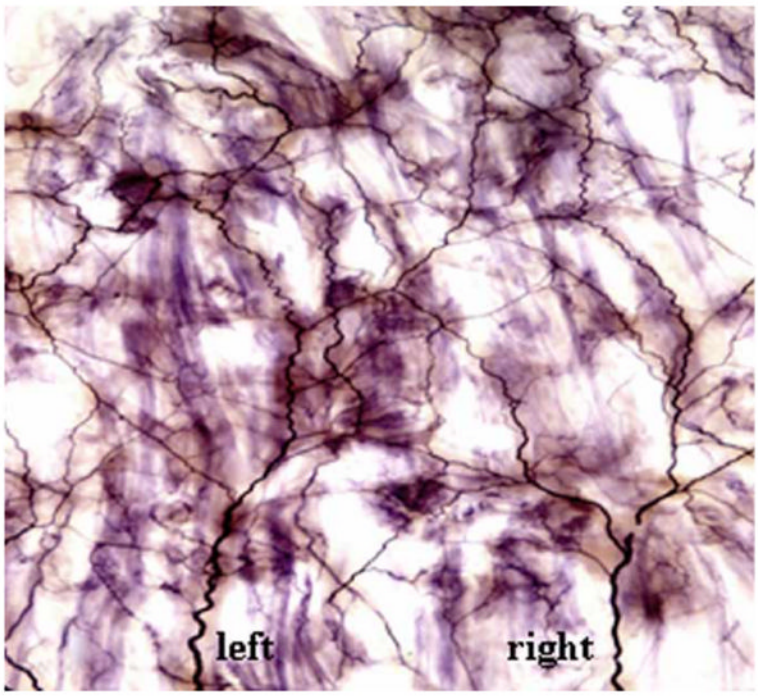

Fig. 9.
Magnification of the rostral compartment within the canine geniohyoid muscle as shown in Fig. 5. Note that this compartment is innervated bilaterally. The nerve branches on both sides cross the midline and connect with each other to form a network. 9 ×.

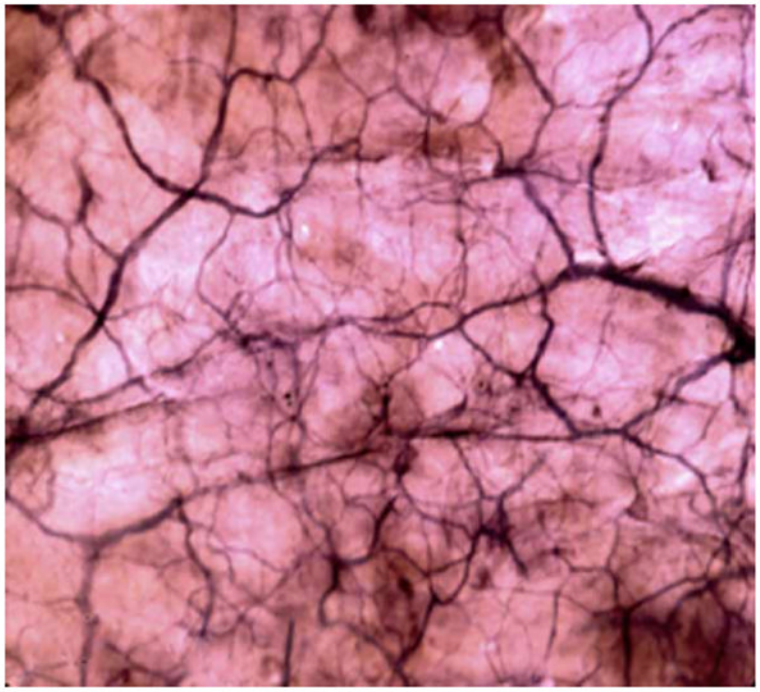

Fig. 10.
Sihler's stained adult human epiglottis. Note that the mucosa covering the laryngeal surface is supplied bilaterally by the internal superior laryngeal nerve. The terminal branches connect with each other to form a dense network. 9 ×.

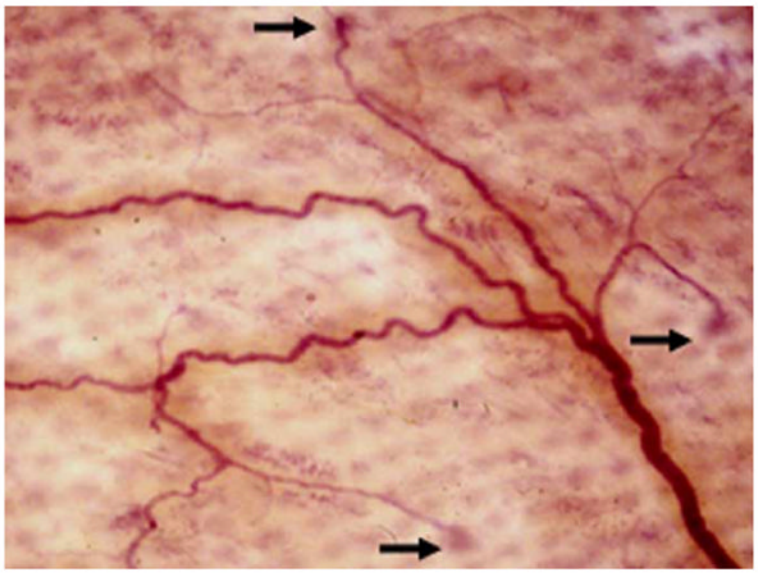

Fig. 11.
Microdissected dorsal mucosa of the anterior canine tongue processed with Sihler's stain showing that the lingual nerve terminals innervate taste buds (arrows). 9 ×.

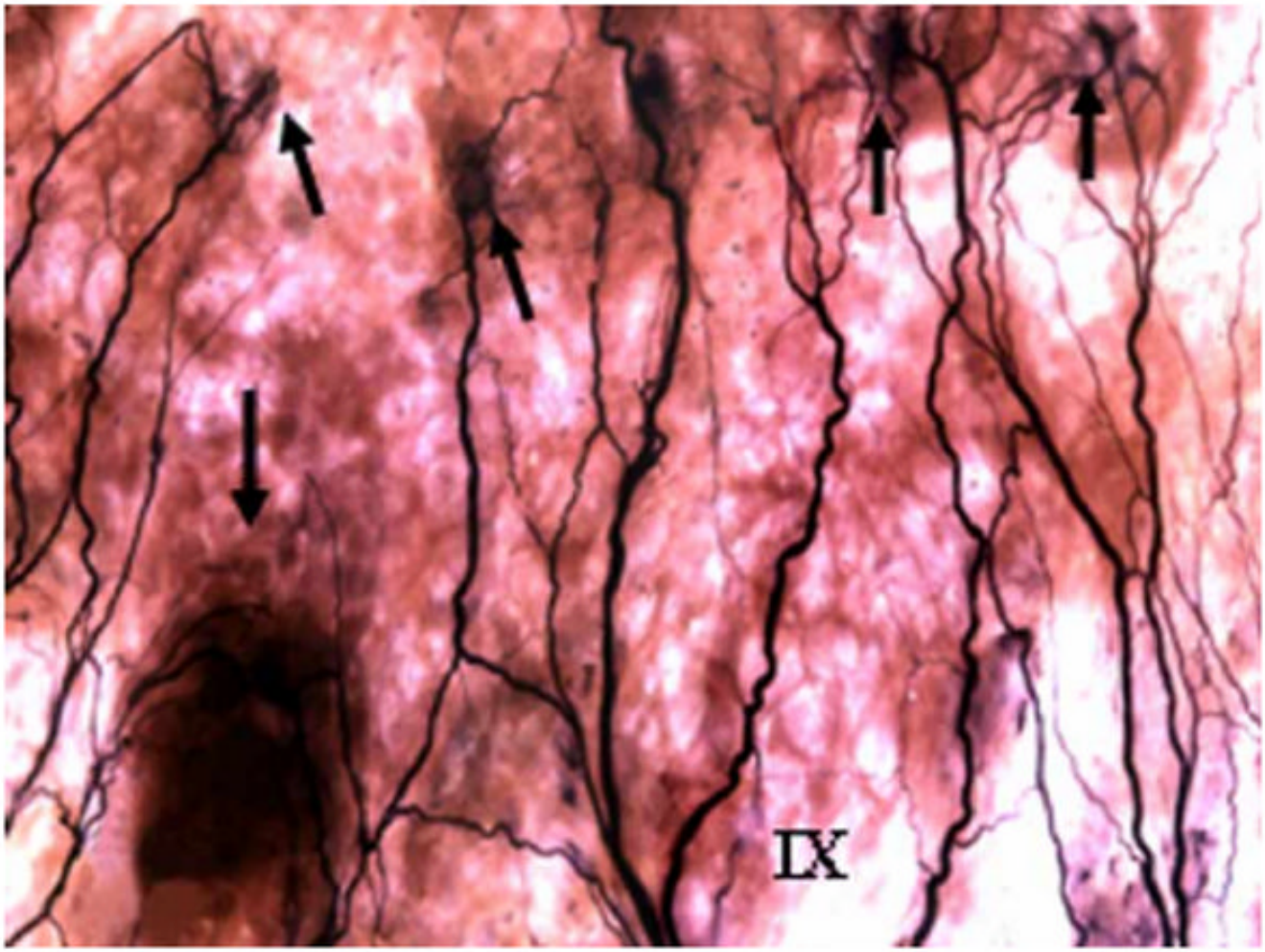

Fig. 12.
Sihler's stained posterior adult human tongue. Note that the glossopharyngeal (IX) nerve terminals innervate the circumvallate papillae (arrows). 9 ×.

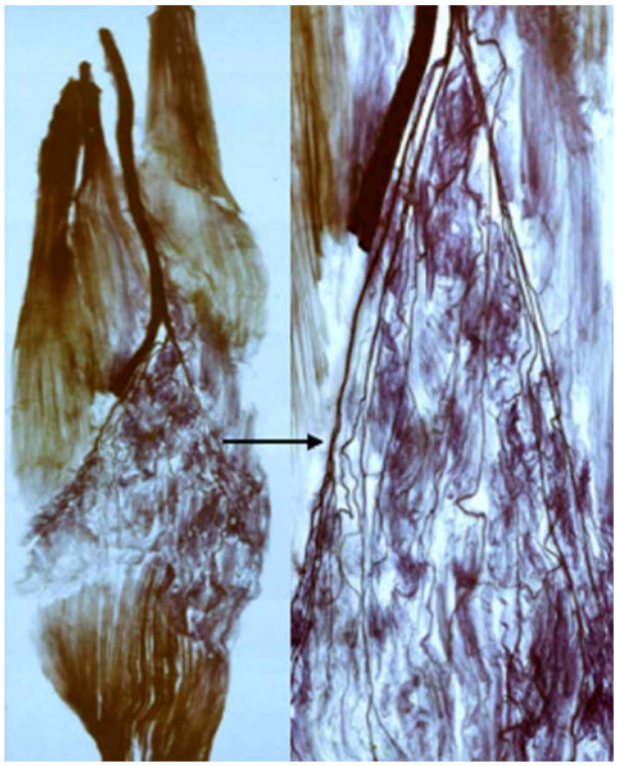

Fig. 13.
An adult human biceps brachii muscle processed with Sihler's stain. Note that Sihler's stain shows the intramuscular nerve branching and distribution in this large human limb muscle.

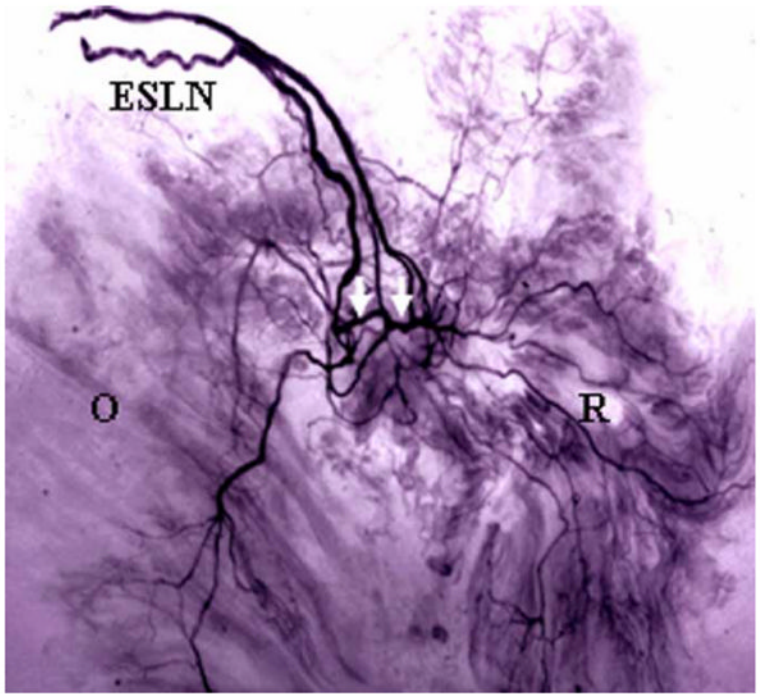

Fig. 14.
Sihler's stained adult human cricothyroid muscle showing the neural connections (arrows) between two primary motor branches derived from a single nerve, the external superior laryngeal nerve (ESLN). Note that both primary nerve branches supply the rectus (R) and oblique (O) bellies of the cricothyroid muscle, respectively. 6 ×.

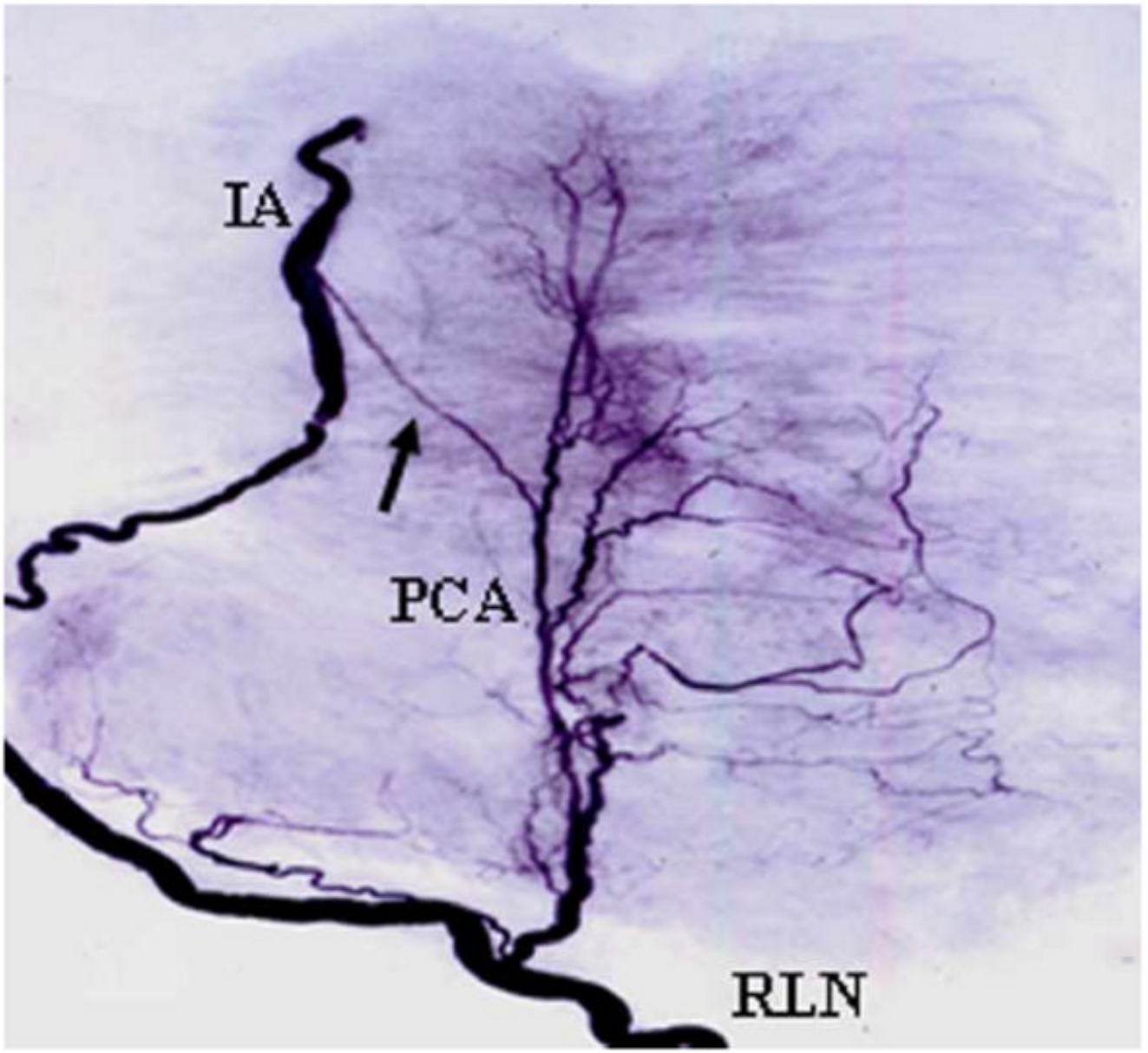

Fig. 15.
Sihler's stained adult human posterior cricoarytenoid (PCA) muscle showing a neural connection (arrow) between two motor nerve branches, posterior cricoarytenoid branch and interarytenoid (IA) branch that are derived from a single nerve, the recurrent laryngeal nerve (RLN). 6 ×.

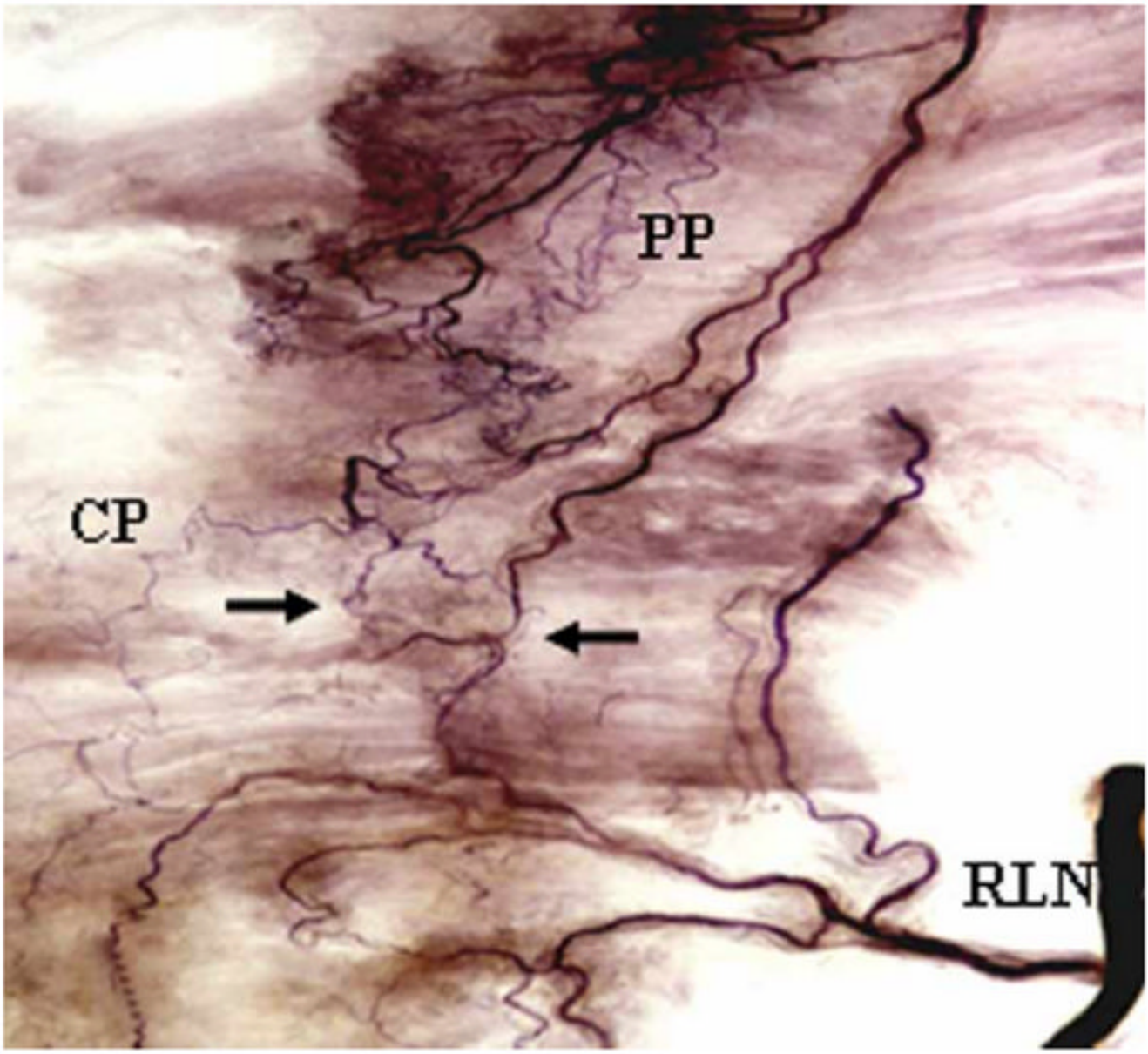

Fig. 16.
Adult human cricopharyngeus (CP) muscle processed with Sihler's stain showing the neural communications (arrows) between two different motor nerves, recurrent laryngeal nerve (RLN) and pharyngeal plexus (PP). 9 ×.

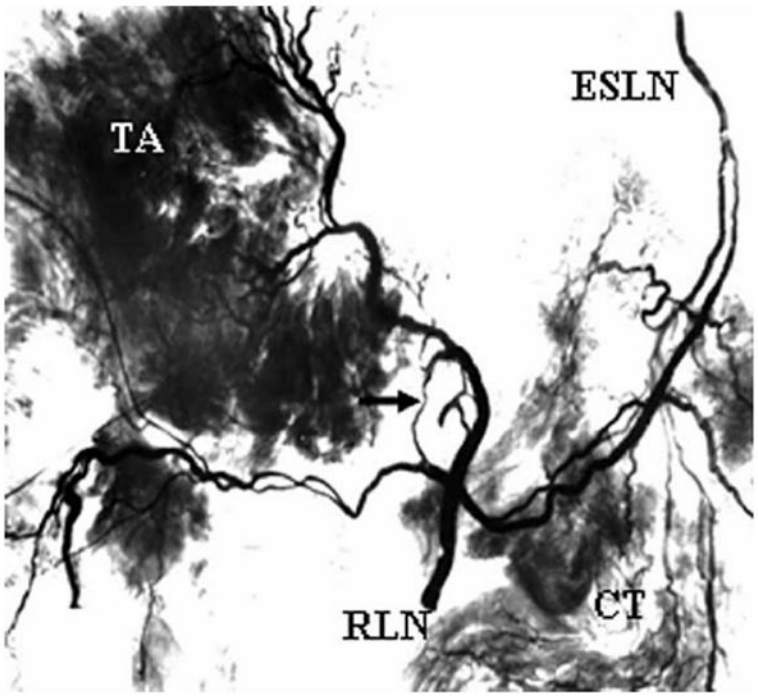

Fig. 17.
Sihler's stained adult human laryngeal muscles showing the neural connection (arrow) between two different motor nerves, external superior laryngeal nerve (ESLN) and recurrent laryngeal nerve (RLN). Note that one of the external superior laryngeal nerve branches passed through the cricothyroid (CT) muscle and gave off a branch to connect with the recurrent laryngeal nerve. TA, thyroarytenoid muscle. 6 ×.

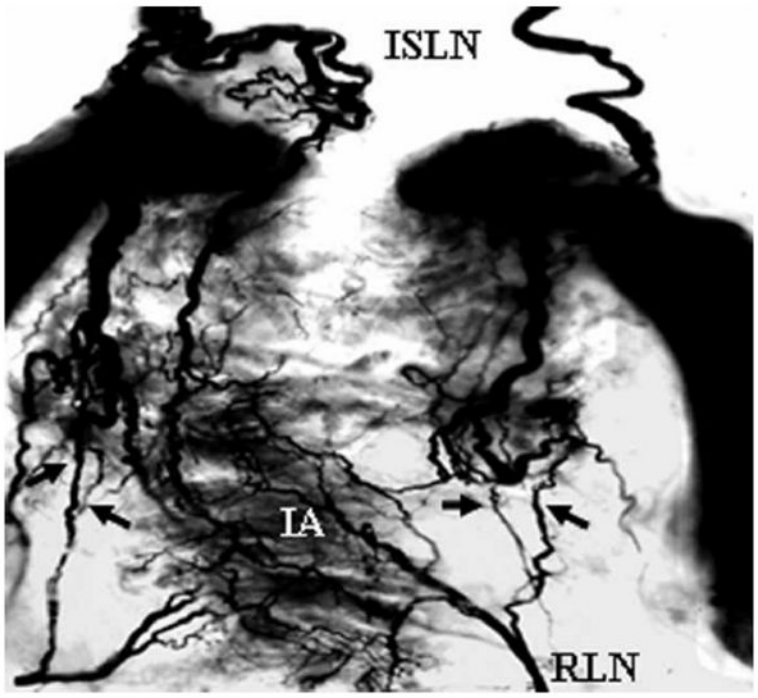

Fig. 18.
Sihler's stained adult human interarytenoid (IA) muscle showing the neural communications (arrows) between a motor nerve, recurrent laryngeal nerve (RLN) and the traditionally described sensory nerve, internal superior laryngeal nerve (ISLN). 6 ×.

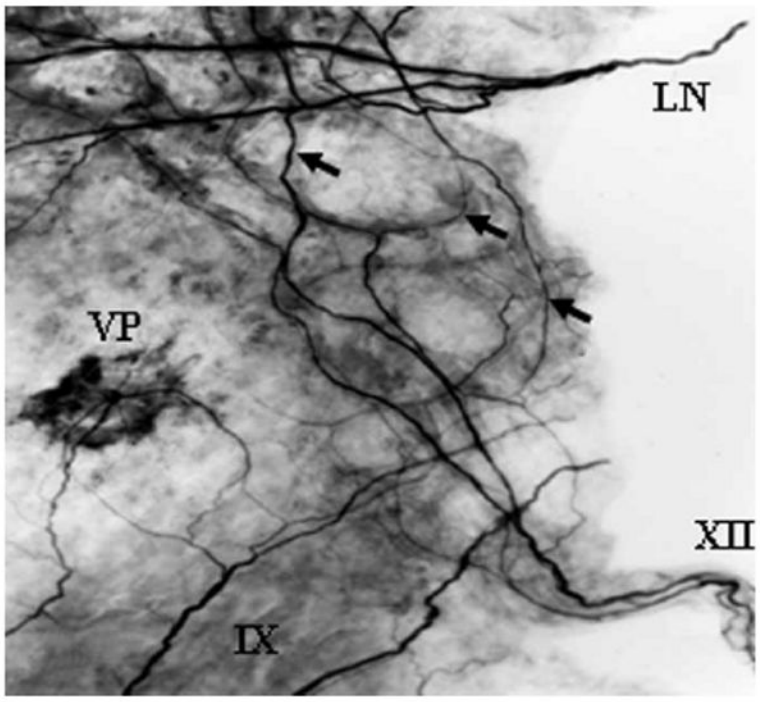

Fig. 19.
Sihler's stained canine posterior tongue illustrating the neural communications (arrows) between a sensory nerve (lingual nerve; LN) and a motor nerve (hypoglossal nerve; XII). IX, glossopharyngeal nerve; VP, circumvallate papillae. 6 ×.

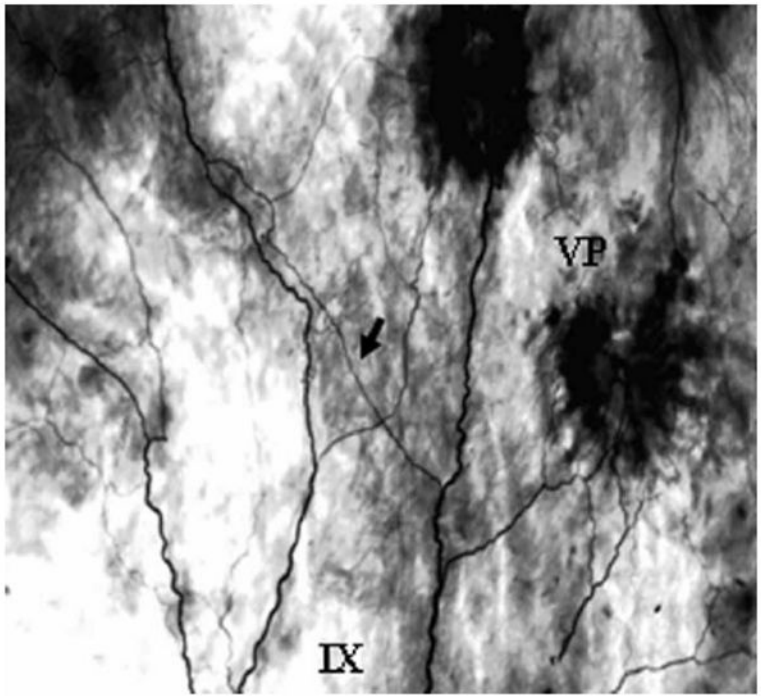

Fig. 20.
Sihler's stained adult human posterior tongue showing the neural connection (arrow) between two major nerve branches derived from a single sensory nerve, glossopharyngeal nerve (IX). VP, vallate papillae. 9 ×.

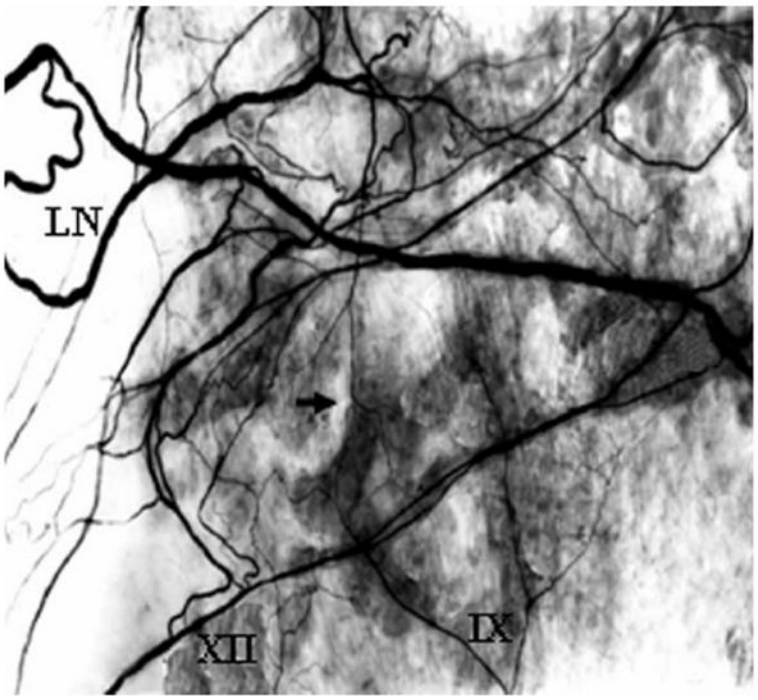

Fig. 21.
Sihler's stained canine posterior tongue showing the neural connection (arrow) between two branches derived from different sensory nerves, lingual (LN) and glossopharyngeal (IX) nerves. 6 ×.

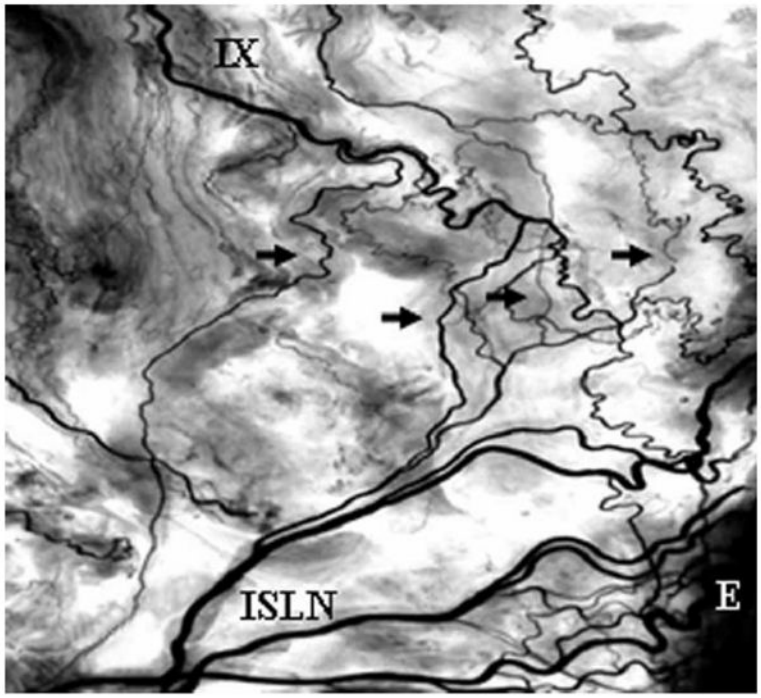

Fig. 22.
Sihler's stained adult human pharynx showing the neural communications (arrows) surrounding the epiglottis (E) between two different sensory nerves, glossopharyngeal (IX) and internal superior laryngeal nerves (ISLN). 9 ×.

Table 1

Distribution of the reports on the use of Sihler's nerve staining technique

Countries	Number of papers	Percentage (%)	Years of publications	Tissues studied
USA	29	60	1987–2008	Human and animal cranial & limb muscles and mucosa; rat skin
Turkey	8	17	2001–2007	Human fetal limb & trunk muscles; rodent extraocular, diaphragm, masticatory & trunk muscles
Singapore	6	13	1997–2007	Human, monkey & rabbit limb muscles; human trunk muscles
The Netherlands	1	2.0	1988	Rat hard palate
Australia	1	2.0	1997	Rat posterior cricoarytenoid muscle
Austria	1	2.0	2001	Human trapezius muscle
Ireland	1	2.0	2003	Rabbit larynx
UK	1	2.0	2005	Pig larynx
Total	48	100		